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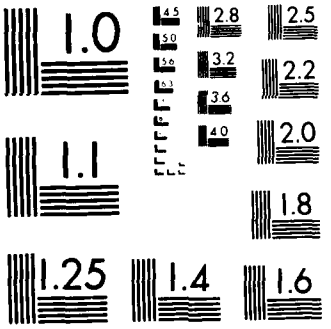

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Yigal H. Ehrlich, Ph.D.

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Four main directions of investigation have been pursued. Progress has been made in each. First, the endogenous protein phosphorylation systems in various subcellular fractions of neural cells differentiated in-culture have been identified and characterized. Second, we have obtained direct evidence for ecto-protein kinase activity at the surface of neural cells and identified its specific protein substrates. Third, a method for preparing and screening monoclonal antibodies against specific neuronal phosphoproteins has been developed and implemented in our laboratory. Finally, studies of the above systems in primary cultures of brain neurons have been initiated. Several manuscripts describing these novel findings are currently prepared in addition to those already published. Studies on the functional role of protein phosphorylation systems in processes underlying neuronal responsiveness have been initiated during year 02 of this project, using both neural cell lines and primary CNS neurons. In year 03 of the project our research focused on the extracellular protein phosphorylation systems of neostriatral cells differentiated in culture.

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Dr. William O. Berry	(202) 767-5021	NL

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FINAL SCIENTIFIC REPORT
FOR GRANT AFOSR 84-0331

Project Title: Role of Protein Phosphorylation in the Regulation of Neuronal Sensitivity.

Principal Investigator: Yigal H. Ehrlich, Ph.D.

Period of Support: September 1, 1984 to February 29, 1988.

(1) Concise Summary

Our research program utilizes neuronal cells grown in culture under defined chemical conditions in experiments designed to determine the role of specific protein phosphorylation systems in molecular mechanisms underlying the processes whereby environmental, hormonal and pharmacological stimulations induce adaptive alterations in receptor sensitivity and in neuronal responsiveness. The specific aims of this project were: (1) Determine the optimal conditions for growth, maintenance, differentiation, and synaptogenesis of neuronal cells in culture. (2) Characterize the intra- and extra-cellular protein phosphorylation systems operating in mature, cultured neuronal cells, and identify their endogenous protein substrates. (3) Purify components of the neuronal-specific protein phosphorylation systems, and raise antibodies against them. (4) Utilize the data-basis, tools, and probes obtained in the studies outlined above in a systematic series of experiments designed to provide direct evidence of causal relationships between the phosphorylation of specific proteins and certain cellular activities underlying adaptive alterations in neuronal function. During the period of this grant we have made progress in each of these lines of investigation. These studies have been carried out with four separate cell systems, each used for a different specific purpose: NG108-15 cells differentiated by dibutyryl cyclic AMP were studied as a model of cells with mature pre-synaptic activity, but devoid of post-synaptic elements. PC-12 cells differentiated by NGF were studied as a model of adrenergic neurons. Platelets were used in our studies as a model system in the investigation of receptor-mediated exocytosis. Finally, and most importantly, we have established in our laboratory the capability and expertise to grow and maintain in culture pure population of primary neurons from defined regions (neostriatum and cortex) of embryonic brain, which undergo synaptogenesis in culture in a defined molecular environment. In each of these cell systems we have obtained direct evidence for the existence of ecto-protein kinase, identified its endogenous protein substrates, and began to investigate their role in several neuronal functions: response to depolarization; calcium fluxes, uptake of norepinephrine; neuronal adhesion mediated by N-CAM; agonist-induced vesicular secretion; neuronal development and synaptogenesis, and molecular events of neuronal signal-transduction associated with synaptic plasticity.

2) Comprehensive List of Research Objectives

A. Establish Differentiated Neuronal Cells Grown in Culture as a model system for studying the role of protein phosphorylation in neuronal function and neuronal adaptation. This research involves establishment of the optimal

conditions for obtaining pure populations of differentiated neural cells, determination of the types of protein kinases located in various subcellular fractions and identification of their specific endogenous protein substrates, comparing differentiated to immature cells by cell free assays and by labeling of intact cells. We have anticipated that in the second half of the grant period the focus of these experiments will shift to studies of functional interactions between protein phosphorylation systems and mechanisms underlying the regulation of receptors, as outlined in the original proposal. This expectation has been fulfilled. We have conducted these functional studies in our laboratory with four complementing model systems: NG108-15 cholinergic cells, PC-12 adrenergic cells, platelets and primary CNS neurons.

B. Detailed characterization of the activity of ecto-protein kinase that we have identified in neuronal cells grown in chemically defined media, and identification of its specific protein substrates localized on the outer surface of the plasma membrane. The second phase of this investigation involved experiments designed to determine the role of ectokinase activity in the increased Ca^{++} uptake induced in neural cells by extracellular ATP. Demonstration of causal relationships in these interactions requires isolation of the ectokinase and preparation of monoclonal antibodies against its substrates, as originally proposed.

C. In addition to the two parallel studies detailed above, we continued with our successful operation of developing monoclonal antibodies to specific phosphoproteins, with emphasis on protein substrates of ectokinase activity; immunizing mice with intact (unlysed) cells and using the ^{32}P-RIA developed in our laboratory for screening of the antibodies.

D. Investigation of the relevance of the molecular events studied under Objectives A,B and C above to processes operating in the Central Nervous System. These studies are carried out with primary cultures of neuronal cells prepared from select brain regions, and then grown and differentiated in culture in a chemically defined medium.

(3) Significance and Accomplishments of the Research.

A large body of evidence has accumulated demonstrating that ATP is stored within synaptic vesicles and secreted in association with classical neurotransmitters at certain synapses and neuromuscular junctions. The finding of transmission systems mediated by ATP and its unhydrolizable analogs has led to the discovery of specific ATP receptors that operate by activating signal transduction processes. In addition, several studies have described neuromodulatory effects of extracellular ATP which require native, hydrolizable ATP. Such modulation was demonstrated both pre- and post-synaptically, but the molecular mechanisms underlying these actions of extracellular ATP have not been elucidated yet. Studies in our laboratory address this issue by investigating the activity of ecto-protein kinases which utilize secreted ATP as a co-substrate in the phosphorylation of proteins located at the outer surface of neuronal cells. In analogy to the well documented role of intracellular protein-kinases in regulating the function of various enzymes, receptors and ion channels, it is anticipated that the identification, characterization, and isolation of extracellular protein kinase(s) and surface phosphoproteins would reveal a new

mode of regulation of synaptic function, cell-cell interactions in the nervous system, neuronal adaptation and synaptic plasticity.

Our approach for investigating extracellular protein phosphorylation systems in the nervous system is based on the study of intact neuronal cells grown and differentiated in-culture in a chemically defined environment. We measure the phosphorylation of surface proteins by radiolabeled ATP added to the extracellualar medium. The location of the protein kinase(s) responsible for this catalytic activity was verified by carrying out experiments designed to fulfill established criteria accepted as evidence for the presence of ecto-enzymes. Our studies focus on the biochemical characterization of neuronal ecto-protein kinase and its specific substrates; and on the use of several model systems of cultured neural cells in the investigation of its functional properties. The involvement of extracellular protein phosphorylation systems in neuronal development and synaptogenesis is studied in our laboratory by following the maturation in culture of embryonic neostriatal and cortical neurons. Vesicular secretion of ATP is measured directly upon depolarization of these CNS cells. Phosphorylation of neuronal cell adhesion molecules (N-CAM's) by extracellualr ATP has provided the first clue for a potential role of this activity in developing neurons. Cloned neural cells of the lines NG108-15 and N1E-115 have provided correlative evidence for the regulation of neuronal calcium-uptake by ecto-protein kinase. Studies of the adrenergic cell PC-12 have implicated the phosphorylation of a surface protein (MW = 39-41K) in high-affinity norepinephrine uptake. Exocytosis of a soluble protein kinase (an exo-kinase) by cells which secrete ATP upon stimulation (neurons and platelets) is studied to provide the basis for additional means of intercellular communication. Finally, the accumulation of relatively high levels of ATP in the synaptic cleft during repetitive stimulation is investigated in studies designed to delineate the role of ecto- and exo-protein kinases in molecular mechanisms underlying neuronal adaptation and synaptic plasticity.

A detailed account of the progress made in our studies supported by grant AFOSR 84-0331 was included in the porposal submitted in request for the continuation of this research. Studies that have been completed during the support period are described in detail in the publications listed in the next section and highlighted in the titles of the listed articles.

(4) Written Publications:

A. Book:

Ehrlich, Y.H., Lenox, R.H., Kornecki, E. and Berry, W. (editors) "Molecular Mechanisms of Neuronal Responsiveness", Volume no. 221 in the series Advances in Experimental Medicine and Biology, Plenum Press, New York (1987), 563 pp.

B. Articles:

Ehrlich, Y.H., Davis, T., Bock, E., Kornecki, E. and Lenox, R.H. Ecto protein kinase activity on the external surface of neural cells. Nature (London) 320:67-69, 1986.

Ehrlich, Y.H. and Kornecki, E. "Extracellular Protein Phosphorylation systems in Cellular Responsiveness", In: Mechanisms of Signal Transduction by Hormones and Growth Factors, G. Sato, W.L. McKeehan, and M. Cabot, Editors, Alan Liss Inc., N.Y., pp. 193-205, 1987.

Hendley, E.D., Whittemore, S.R., Chaffee, J.E. and Ehrlich, Y.H. Regulation of Norepinephrine Uptake by Adenine Nucleotides and divalent cations: Role for extracellular protein phosphorylation. Journal of Neurochenistry, 50:263-273, 1988.

Ehrlich, Y.H., Snyder, M. Kornecki, E., Garfield, M.K. and Lenox, R.H. Regulation of Signal Transduction System in Neuronal Cells by Extracellular ATP. Journal of Neurochemistry, 50:295-301, 1988.

Zhang, J., Kornecki, E., Jackman, J. and Ehrlich, Y.H. Secretion of ATP and Phosphorylation of Surface Proteins in Primary CNS Neurons Differentiated in Culture. Brain Research Bull. (in-press).

Kornecki, E. and Ehrlich, Y.H. Neuroregulatory and Neuropathological Actions of the Ether-Phospholipid Platelet Activating Factor (PAF). (in-press).

C. Manuscripts in preparation:

Babinska, A., McCullum, R. and Ehrlich, Y.H. Characterization of GTP-Preferring Protein Phosphorylation Systems in Brain Synaptic Membranes.

Ehrlich, Y.H., Kornecki, E. and Cierniewski, C.S.
Platelet Activation Mediated by a Phosphoprotein Substrate of an Ecto-Protein Kinase.

Kornecki, E. and Ehrlich, Y.H. Platelet Activation and Inhibition by Unique Monoclonal Antibodies to Surface Proteins.

Davis, T.B and Ehrlich, Y.H. Specific Alterations in Endogenous Protein Phosphorylation Systems during Differentiation of NG108-15 cells in culture.

Ehrlich, Y.H., Jenny, R.J., Kornecki, E. and Mann, K.G. Phosphorylation of Coagulation Factor V/Va by an Exo-Protein kinase Secreted from Activated Human Platelets.

Bock, E., Ehrlich, Y.H. et al. Endogenous phosphorylation of N-CAM and NG-CAM by Ecto-protein kinase of Neuronal Cells in Primary Culture.

Ehrlich, Y.H. Naturally Occurring Complexes Containing Endogenous Phosphorylation Systems in the Cytoplasm of Brain Tissue.

D. Abstracts:

Davis, T.B., Kornecki, E., Lenox, R.H. and Ehrlich, Y.H. Neuroblastoma x glioma hybrid cells differentiated in culture: A model for studying the function of neuronal phosphoproteins. Society for Neuroscience, (1), p. 196, 1984.

Ehrlich, Y.H., Davis, T.B., Wesson, J., Kornecki, E. and Lenox, R., Specific substrates for ecto-protein kinase activity on the external surface of neuroblastoma x glioma cells. Trans. Am. Soc. Neurochem., 1985.

Ehrlich, Y.H., Davis, T.B., Wesson, J., Kornecki, E. and Lenox, R.H. Ecto-protein kinase and specific substrates on the external surface of neural cells. J. Neurochem. 44: suppl. 530, 1985.

Ehrlich, Y.E., Kornecki, E., Jenny, R., Cierniewski, C.S., and Mann, K.G. (1986) Regulation of platelet activation and blood coagulation by extracellular protein phosphorylation systems. Blood, 68: Suppl. 1, 315a.

Zhang, J., Kornecki, E., Jackman, J., and Ehrlich, Y.H. (1987) Depolarization-induced secretion of ATP from mature CNS neurons in primary culture. J. Neurochem., Vol. 48 Supplement.

Kornecki, E., Neel, D., Parsons, R. and Ehrlich, Y.H. (1987) Neuronal stimulation by the alkyl-ether phospholipid, platelet activating factor (PAF-acether). J. Neurochem., Vol. 48 Supplement.

Ehrlich, Y.H. (1987) Extracellular protein phosphorylation systems in the regulation of neuronal function. Invited symposium presentation at the Joint meeting of the International and American Societies for Neurochemistry.

Ehrlich, Y.H., Snider, R.M., Garfield, M.G., Kornecki, E. and Lenox, R.H. Modulation of neuronal signal transduction by extracellular ATP. J. Neurochem., Vol. 48 Supplement 5113B (1987).

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Ehrlich, Y.H. and Kornecki, E. (1988) Ecto- and exo-protein kinases in human platelets. Trans. Amer. Soc. Neurochem. 19, 184.

Ehrlich, Y.H. , Galbraithe, I., Jackman, J. and Kornecki, E. Evidence for and characterization of ecto-protein kinase activity in primary CNS neurons. Society for Neuroscience Abstracts. Vol. 14, 1988.

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